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Use of a Mouse Model of Experimentally Induced Endometriosis to Evaluate and Compare the Effects of Bisphenol A and Bisphenol AF Exposure

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Background: Endometriosis is a gynecological disease affecting 1 in 10 women of reproductive age. Endometriosis incidence has risen; however, whether this rise is due to disease awareness or environmental contamination is not known.

Objective: The objective of this study was to determine if bisphenol A (BPA) or bisphenol AF (BPAF) potentiate the development of endometriosis and if hormonal status alters how toxicant exposure affects disease.

Methods: A mouse model of endometriosis, where minced uterine tissue is injected into the peritoneal cavity of a host mouse, was used to examine the effects of BPA and BPAF on endometriosis lesion development in ovariectomized and hormonally intact mice. BPA and BPAF were delivered through diet to include no-observed-adverse-effect-level (NOAEL) and the low-observed-adverse-effect-level (LOAEL) exposure levels. After six weeks (at necropsy), lesions, ovaries, and blood were collected to examine characteristics, gene expression, and hormonal regulation.

Results: BPA and BPAF treatments affected endometriosis in a manner specific to dose and hormonal status of the host mouse. Estrogen and endometriosis-mediated differences in lesion target gene expression also depended on hormonal status. In intact mice, ovarian steroidogenic pathways were disrupted, progesterone levels were lowered, and atretic oocyte numbers were higher with toxicant exposure. BPAF, more so than BPA, resulted in more endometriosis lesion growth, but both toxicants disrupted normal ovarian signaling.

Conclusion: These findings further our understanding of the effects and hormonal impacts of BPA and BPAF on endometriosis perturbation in ovariectomized and hormonally intact mice. BPAF appeared to be similar if not more estrogenic than BPA and may be affecting an environmental contribution of the increased incidence of endometriosis. https://doi.org/10.1289/EHP3802

Introduction

Endocrine-disrupting chemicals (EDCs) are environmental toxicants that interfere with the endocrine system and can do so at various doses. Essentially, these EDCs disrupt normal hormone function by either mimicking or blocking hormones and cause adverse health effects (Gore et al. 2015a, 2015b). Much like hormones, EDCs can be functional at low doses; therefore, they have a great impact on health and disease (Schug et al. 2011). EDCs are pervasive and known to affect common diseases, such as breast cancer, diabetes, obesity, and heart disease (Chen WY et al. 2016; Rachoń 2015). The bisphenols are EDCs known to act through the estrogen receptors (ERs) to regulate downstream signaling and gene expression responses (Lei et al. 2017; Li et al. 2013; Matsushima et al. 2010). Bisphenol A (BPA), used to make polycarbonate plastics and epoxy resins, is estrogenic and is being phased out of currently manufactured polycarbonate plastics (Vandenberg et al. 2013). Instead, Bisphenol AF (BPAF), a fluorinated homologue of BPA, is one of the alternatives used as a BPA replacement for cross-linking. Mass quantities of BPAF are now being produced in the United States annually, and recently, BPAF was detected in food, dust, sediment, and municipal sewage sludge (Chen D et al. 2016; Pelch et al. 2017). As with BPA, studies demonstrate BPAF acts estrogenically through ERα and ERβ (Li et al. 2012, 2013, 2018; Matsushima et al. 2010) and that BPA and BPAF have acted agonistically and antagonistically on ERs and ER downstream functionality (Pelch et al. 2017). BPAF is more estrogenic than BPA (Li et al. 2012, 2013); therefore, BPAF may have the potential to be a contributing factor in the higher incidence of endometriosis.

Endometriosis affects ∼10 million women and adolescents of reproductive age, and costs associated with disease are upwards of $120 billion in the United States alone, using the most recent direct and indirect cost estimates of disease (Soliman et al. 2018). Endometriosis occurs when functioning endometrial tissue attaches outside of the uterine cavity (Sampson 1927; Syrop and Halme 1987). This attachment is a result, in part, of retrograde menstruation, in which menstrual tissue flows back through the fallopian tubes and into the peritoneal cavity (Sampson 1927; Syrop and Halme 1987). Approximately 90% of women experience retrograde menstruation, but only ∼10% will develop endometriosis (Sampson 1927; Syrop and Halme 1987). Endometrial lesions are found attached to sites in the peritoneal cavity, such as the fallopian tubes, ovaries, peritoneal wall, and bowel. Symptoms include painful menstruation, pain with intercourse, infertility, and chronic pain (Giudice and Kao 2004). A true diagnosis of disease is achieved only through laparoscopic surgery, and most treatments (e.g., birth control pills and pain medication) are treating only symptoms, not the disease (May et al. 2010). The pathogenesis of disease is largely unknown; however, the proliferation of endometriosis is known to be hormone dependent (Giudice and Kao 2004). The incidence of endometriosis has risen from approximately 3.3% in the 1970s (Houston et al. 1987) to a current incidence of 10–15% and as high as 70% in women with chronic pelvic pain (Fuldeore and Soliman 2017; Parasar et al. 2017); however, whether this increased incidence is due to disease awareness or environmental toxicant exposure is not understood (Anger and Foster 2008).

In previous studies, EDCs have been associated with the progression of endometriosis. In particular, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was the first reported EDC to lead to the development of severe endometriosis in rhesus monkeys (Rier and Foster 2003; Rier et al. 1993, 2001). Currently, BPA is one of the best-known and most-pervasive EDCs (Rachoń 2015). Women with endometriosis had more abundant serum levels of

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BPA than did women without disease (Cobellis et al. 2009), and a population-based study reported that higher urinary BPA levels are associated with a greater risk of nonovarian pelvic endometriosis (Upson et al. 2014). Based on this knowledge, the greater production and exposure to BPAF in the environment, and the previous work that demonstrated BPAF is more estrogenic than BPA, we hypothesized that greater disease burden of endometriosis will be observed with BPAF treatment in comparison with BPA and vehicle treatments.

In this study, we used a mouse model of endometriosis to determine the effects of BPA or BPAF on the perturbation of disease and ovarian function. Both ovariectomized and hormonally intact mice were used to examine the effects of BPA or BPAF without or with hormonal interactions, respectively. After six weeks of BPA or BPAF treatment, we, to our knowledge, for the first time, directly compared the effects of BPA or BPAF on the potentiation of endometriosis using ovariectomized and hormonally intact models of endometriosis.

Materials and Methods

Animal Husbandry

All animal studies were approved by the University of Cincinnati's Institutional Animal Care and Use Committee (IACUC) and followed the Panel on Euthanasia of the American Veterinary Medical Association. Mice per group were based on sample-size calculations made using preliminary data that compared treated vs. control and were conducted using SAS Proc Power (SAS Institute, 2008). A minimum of 6 mice per group were used, with a maximum of 25 per group in the negative and positive control groups, as they were included with each experiment. Sexually mature transgenic C57BL/6-Tg(UBC-GFP)30Scha/J (GFP) (IMSR Catalog # JAX:004,353, RRID: IMSR_JAX:004,353) and 6- to 8-wk-old healthy wild type C57BL/6J (IMSR Catalog # JAX:000,664, RRID: IMSR_JAX:000,664) female mice were purchased from Jackson Laboratories. Mice weighed 18–22 grams. Animals were randomly group-housed five to a cage in BPA-free conditions according to Kendziorski and Belcher (2015) and were given ear-punch designations upon receipt. Bisphenol A and analogs are known to have short urinary half-lives of approximately 4 h; therefore, incorporation of the EDCs into the diet were used to ensure constant daily exposure (Kendziorski and Belcher 2015; Stahlhut et al. 2009).

Wild-type host animals were fed a defined casein-based phytoestrogen-free diet (Product # D10010501, Research Diets) without supplement (vehicle) or homogenously incorporated by the manufacturer with EE [1,3,5(10)-estratrien-17α-ethinyl-3,17β-diol; CAS No. 57-63-6; Batch B1460; Steraloids Inc.], BPA [2,2-bis(4-hydroxyphenyl)propane; CAS No. 80-05-7; Lot AOHOK; National Toxicology Program], or BPAF [2,2-Bis(4-hydroxyphenyl)hexafluoropropane; CAS No. 1,478-61-1; Lot 5G6DC-TJ; TCI America] (Figure 1A). EE diet was produced with final concentration of 0.01 ppm, which corresponded to an approximate daily dose of 0.001 mg/kg/day (Kendziorski and Belcher 2015). Doses were calculated to incorporate BPA or BPAF into the diet for animal exposure to encompass the Environmental Protection Agency (EPA) advised no-observed-adverse-effect-level [NOAEL (5 mg/kg/day)] and the low-observed-adverse-effect-level (LOAEL [50 mg/kg/day]) of BPA (EPA 2010). No NOAEL or LOAEL is set for BPAF. According to quantified oral BPA exposures and the levels found in serum of nonhuman primates and mice, it was estimated that humans receive a dose greater than 500 μg/kg/d (Taylor et al. 2011; Vandenberg et al. 2010a, 2010b, 2013; vom Saal et al. 2010). Based on the serum concentrations described; the linearity of BPA pharmacokinetics; and similar metabolic rates in mice, nonhuman primates, and humans, we back calculated the human exposure levels to be 0.1–86 mg/kg/d with an approximate average being about 2.4–9 mg/kg/day, based on a number of published sources (Vandenberg et al. 2007, 2010a, 2010b, 2013; vom Saal et al. 2007, 2010). This human range in exposure, the LOAEL, and the NOAEL guided the doses chosen for this study. The diets were formulated based on our mice eating approximately 2 g/day and weighing 20 g. The calculation given by Research Diets Inc. is: Diet Dose = (Single Daily Dose × Body Weight)/Food Intake. This calculation was used to determine the approximate single daily dose to be in a range encompassing human exposure, the NOAEL, and the LOAEL. BPA or BPAF was incorporated into the food at 30, 300, or 900 ppm for approximate daily doses of 3, 30, or 90 mg/kg/day, respectively. Weekly food consumption and body weights were tracked on a per cage and per animal basis, respectively. Average daily food consumption rates were extrapolated for each mouse (2.35 ± 0.09 g/day).

All mice were randomly assigned to treatment groups for each experimental replicate. For ovariectomized mouse experiments, groups consisted of vehicle ($n = 25$), EE ($n = 25$), BPA 30 ppm ($n = 15$), 300 ppm ($n = 9$), and 900 ppm ($n = 9$), and BPAF 30 ppm ($n = 12$), 300 ppm ($n = 6$), and 900 ppm ($n = 9$). For intact mouse experiments, groups consisted of vehicle ($n = 7$), EE ($n = 10$), BPA 30 ppm ($n = 10$), 300 ppm ($n = 9$), 900 ppm ($n = 10$), and BPAF 30 ppm ($n = 10$), 300 ppm ($n = 10$), 900 ppm ($n = 9$). For reproducibility and direct comparisons, every surgical group contained vehicle- and EE-treated animals, groups containing ovariectomized and intact animals were run in parallel, and each dose of BPA was run in parallel to the corresponding dose of BPAF. Host animals were allowed to acclimate to the diet 7 d prior to endometriosis surgery with access to food and water *ad libitum*, and they remained on the diet throughout the remainder of the study (6 wk after initiation of endometriosis). Endometriosis was induced according to our established protocol (Burns et al. 2012, 2018) in both ovariectomized and hormonally intact mice between 0830 to 1230 in the surgical suite of the animal facility at the University of Cincinnati College of Medicine. Briefly, donor GFP female mice were primed 41 h before uterine harvest with pregnant-mare serum gonadotropin (5 IU). After euthanasia, the uterus from the donor GFP female mouse was removed *en bloc* and cleaned of excess tissue. The outer myometrial layer was peeled away, rinsed in phosphate buffered saline (PBS), opened longitudinally, and then minced into ≤1.5 mm pieces. Concurrently, recipient wild-type mice were anesthetized with isoflurane/oxygen and given buprenorphine (0.1 mg/kg) subcutaneously for pain. A right dorsolateral incision (<5 mm) was made in the skin and peritoneal cavity, and the minced donor uterus was injected into the peritoneal cavity in a suspension of 500 μL PBS. The peritoneal cavity was closed, and the skin incision was closed with 9 mm clips. In the ovariectomized experimental model of endometriosis, 1 wk prior to establishing endometriosis, host mice were ovariectomized between 0830 and 1230 for clearance of endogenous hormones (Figure 1B), and the host mice began one of the diets detailed above. In the hormonally intact experimental model of endometriosis, 1 wk prior to establishing endometriosis, host mice were randomly assigned to one of the diets detailed above (Figure 1C). Endometriosis was initiated randomly, and the surgeons (SAL and KAB) were blinded to treatment group. Once endometriosis was initiated, all mice were monitored daily for 6 wk until harvest. For intact mice, estrous cycle progression was evaluated daily 4 wk after the initiation of endometriosis, via vaginal lavage and microscopic analysis as previously described, until necropsy (Allen 1922; Jayes et al. 2014). Smears were scored as follows: proestrus (P) contained primarily nucleated epithelial cells with no or few cornified cells, estrus (E) consisted of mostly cornified cells, metestrus (M) contained epithelial cells with leucocyte attacks, and diestrus (D) consisted of

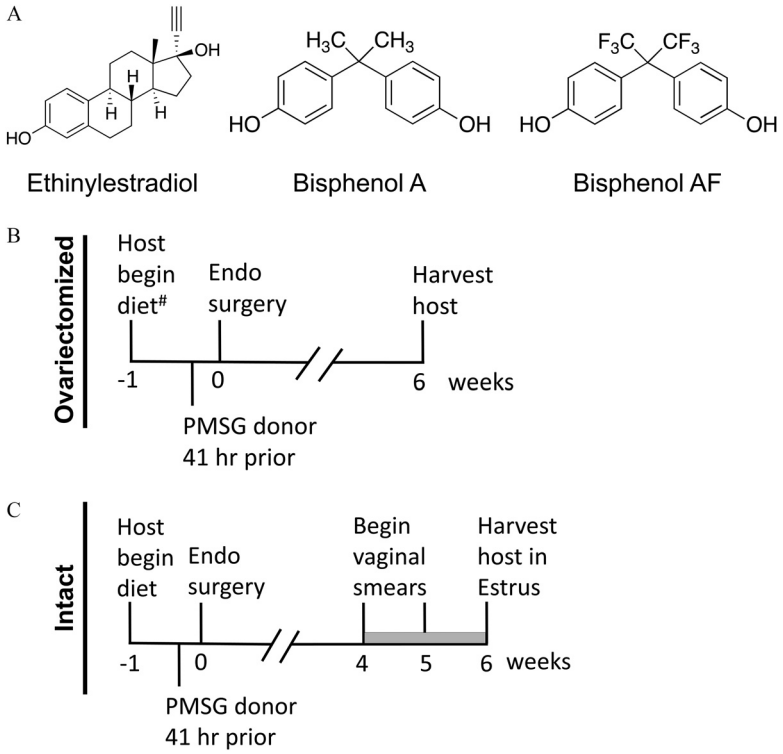

Figure 1. Chemical structure of ligands and endometriosis experimental design. (*A*) Chemical structure of ethinylestradiol, bisphenol A, and bisphenol AF. (*B*) Schematic of ovariectomized experiment. One week prior to inducing endometriosis, host animals were ovariectomized (indicated by #) and began the study diet [containing vehicle (veh), Ethinylestradiol (EE), Bisphenol A (BPA), or Bisphenol AF (BPAF)]. Forty-one hours prior to endometriosis (Endo) surgery, donor mice were primed with pregnant mare serum gonadotropin (PMSG, 5 IU). On the day of surgery, donor mice were euthanized, and recipient mice were injected with minced donor uterine tissue. Mice continued on the study diet for 6 wk, after which time all mice were euthanized. (*C*) Schematic of intact experiment. One week prior to inducing endometriosis, host animals began the study diet (containing veh, EE, BPA, or BPAF). Forty-one hours prior to Endo surgery, donor mice were primed with PMSG (5 IU). On the day of surgery, donor mice were euthanized, and recipient mice were injected with minced donor uterine tissue. Mice continued on the study diet for 6 wk; however, two weeks prior to euthanasia, daily vaginal smears were evaluated, and mice were euthanized in estrus. Shaded area = daily vaginal smears.

few cells that were leucocytes with some rounded or cornified epithelial cells.

Vaginal cytology was read and confirmed by at least two people (RLJ, SAL, JAK, ADG, or KAB) blinded to animal treatment. Animals were euthanized in estrus unless there was no sign of cycling, in which case, the animals were euthanized six weeks after initiation of endometriosis. Animals were excluded from analysis if they experienced extensive barbering, experienced uterine tissue injection failure, or showed signs of sickness; these animals are not included in our *n* numbers. Individuals performing necropsies (SAL, RLJ, KAB) were blinded to animal treatment, dose, and animal hormonal status. At harvest, endometriosis lesions were located, counted, pooled together from each animal for weight, individually measured for volume using microcalipers, and either flash frozen or fixed in 10% formalin. If lesions were large enough, they were divided into two parts, one of which was flash frozen and one of which was fixed in 10% formalin. Ovaries from the intact mouse model were harvested and weighed. One ovary from each mouse was flash frozen, and one ovary was fixed in 10% formalin. Representative lesions from all locations in the peritoneal cavity and ovaries were used for further experimental analysis.

RNA Isolation and Real-Time PCR

Frozen endometriosis lesions from all locations in the peritoneal cavity and ovaries from mice were pulverized on dry ice, and RNA was isolated using TRIzol per manufacturer's protocol (Invitrogen). cDNA was synthesized and analyzed by real-time PCR using Fast SYBR (Burns et al. 2012). Relative transcript levels were quantified in comparison with vehicle group and normalized to *pL7* according to Pfaffl (2001). See Table S1 for primer sequences [Sigma-Aldrich or Thermo Fisher Scientific designed using PrimerBot (http://primerbot.duhs.duke.edu/)].

Immunohistochemistry

Formalin-fixed, paraffin-embedded lesions from all locations throughout the peritoneal cavity were sectioned at 5 micrometers (Histocut 820-II, Reichert-Jung) onto charged slides (Thomas Scientific, Catalog #1158B91), deparaffinized in xylenes, and hydrated through graded alcohols. Immunohistochemistry for Ki67 and cleaved caspase 3 were performed according to Hewitt and Korach and Troja et al., respectively (Hewitt and Korach 2011; Troja et al. 2014). Anti-Ki67 (1:150; Abcam Catalog #ab16667, RRID:AB_302459) and anticleaved caspase 3 (1:500; Cell Signaling Technology Catalog #9,664, RRID:AB_2070042) antibodies were diluted in blocking solution (normal goat serum made according to manufacturer's instructions; Vector Laboratories Catalog #PK-6,101, RRID:AB_2336820) and incubated overnight at 4°C (Table 1). Rabbit normal immunoglobulin (Abcam Catalog #ab172730, RRID:AB_2687931) was used as a negative control and diluted 1:20,000 in phosphate buffered saline with 0.1% Tween-20, then further diluted to 1:150 or 1:500 (Table 1). Protein localization was visualized using 3,3′-Diaminobenzidine (Vector Laboratories Catalog #SK-4,100, RRID:AB_2336382). Sections were counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylenes, and mounted with Permount.

Table 1. Antibody table.

Antibody	RRID	Manufacturer	Cat#	Dilution
Anti-Ki67	AB_302459	AbCam	ab1667	1:150
Anti-Cleaved caspase 3	AB_2070042	Cell Signaling	9664	1:500
Rabbit IgG (1:20,000 dilution)	AB_2687931	AbCam	ab172730	Negative Control 1:150/1:500

Sections were imaged at 100x and 400x using a Nikon TE300 microscope and Leica DFC450c camera with Leica Application Suite X 3.0 software (Leica LAS AF Image Acquisition Software, RRID:SCR_013673). Three individual researchers who were blinded to animal status and treatment scored each lesion section independently based on the observed number of positive Ki67 stained epithelial cells within the entire tissue section. The epithelial proliferation scale was as follows: minimal (0–10%), low (11–30%), moderate (31–50%), high (51–70%), strong (71–90%), and maximum (91–100%). The three blinded scores were averaged to obtain a score for each sample. For each category, a numerical value was assigned. Each sample was scored by RLJ, SAL, ADG, and KAB; these scores were averaged to obtain a final score for each sample. The final scores of all samples were then averaged to obtain the final overall score for each treatment group. Similarly, the level of apoptosis was scored for each lesion section; individual researchers blinded to the animal status and treatment (RLJ, SAL, ADG, KAB) scored the entire lesion cross-section at 400× for relative staining intensity of positive epithelial cleaved caspase 3 staining. The epithelial apoptosis scale was as follows: low (1–5 cells), low/moderate (6–10 cells), moderate (11–20), moderate/strong (21–30), strong (31 +). For each category, a numerical value was assigned. Each sample was scored by RLJ, SAL, ADG, and KAB; these scores were averaged to obtain a final score for each sample. The final scores of all samples were then averaged to obtain the final overall score for each treatment group. Inter-observer variability was minimized by using a series of example photomicrographs to represent each score on the scales noted above for each stain; if scoring discordance was found, all researchers rescored the full section.

Follicle Counts

Formalin-fixed, paraffin-embedded ovaries were sectioned at 5 micrometers onto charged slides, deparaffinized in xylenes, and hydrated through graded alcohols. Sections were stained with hematoxylin and eosin, followed by dehydration through graded alcohols, cleared in xylenes, and mounted with Permount™. Whole ovary cross-sections were counted for the presence of primordial follicles, primary follicles, secondary follicles, ovulatory/antral follicles, corpora lutea, and dead or atretic follicles by researchers (RLJ, confirmed by KAB) who were blinded to animal status and treatment. For each fully effaced ovarian cross-section, a minimum of five serial sections were counted for follicle presence. The final follicle count number for each ovary was acquired by averaging the follicle counts across all cross-sections. Following Patel et al., follicle types and counts were identified (Patel et al. 2017). Succinctly, primordial follicles enclosed an oocyte encircled by a single layer of squamous granulosa cells; primary follicles enclosed an oocyte encircled by a single layer of cuboidal granulosa cells; secondary follicles enclosed an oocyte encircled by at least two layers of cuboidal granulosa cells and theca cells; ovulatory or antral follicles enclosed an oocyte encircled by multiple layers of cuboidal granulosa cells with a fluid-filled antral space; corpus luteum represent the remains of a follicle after ovulation; and dead or atretic follicles enclosed an oocyte encircled by an abnormal configuration of granulosa and theca cells (Patel et al. 2017).

ELISA Hormone Assay

At necropsy, blood was removed from the descending aorta with syringes coated in ethylenediaminetetraacetic acid (EDTA) and was centrifuged at 8,000 rpm for 7 min to obtain plasma. The plasma was flash frozen until further analysis. The three hormones tested were estradiol, follicle stimulating hormone, and progesterone. Each analysis was conducted according to the manufacturer's protocol (Antibodies-online; Estradiol product number ABIN1888500, Follicle Stimulating Hormone product number ABIN1873388, and Progesterone product number ABIN5523586). A dilution series experiment for each hormone determined the correct ratio of plasma to sample buffer (estradiol, 1:5; follicle stimulating hormone, 1:2; progesterone, 1:4).

Statistical Analysis

One-way ANOVA with Tukey's post-test was used to compare lesion and ovary data among vehicle, BPA, BPAF, and EE groups. Further comparison of vehicle to individual treated group were analyzed by a one-tailed unpaired *t*-test. These analyses were performed using GraphPad Prism version 7.02 (Graphpad Prism, RRID:SCR_002798). Level of significance was set at $p < 0.05$. Histograms are presented as mean plus standard error of the mean (SEM).

Results

Lesion Characteristics in BPA- and BPAF-Treated Mice

Lesion number is an indicator for disease establishment. Similar to our previous observations (Burns et al. 2012, 2018), estrogen did not alter the number of lesions in ovariectomized mice. In comparison with control, total lesion numbers from BPA or BPAF-treated groups were not different regardless of toxicant dose (Figure 2A).

Another aspect of endometriosis disease is lesion growth. In response to exogenous treatment, we examined pooled lesion weight and individual lesion volume from treated animals in comparison with vehicle treated animals to elucidate the role of BPA or BPAF on the potentiation of endometriosis lesion growth. As expected, the total lesion weight was greater in ovariectomized mice exposed to EE in comparison with vehicle treatment (Figure 2A). There were no significant differences in lesion weight for mice exposed to BPA 30, 300, or 900 ppm and BPAF 30 and 300 ppm in comparison with those animals exposed to vehicle treatments. Mice exposed to BPAF 900 ppm exhibited significantly greater lesion weight in comparison with those animals with vehicle treatment (Figure 2A).

As in our previous observations (Burns et al. 2012, 2018), mice typically develop a lesion at the injection site [akin to endometriosis found at cesarean scars in women (Khachani et al. 2017)] and BPA was shown to be associated with nonovarian pelvic endometriosis (Upson et al. 2014); therefore, we subanalyzed lesion volumes that were found distal to the injection site. In ovariectomized mice, distal lesion volumes from EE, BPA 300, and 900 ppm, and BPAF 900 ppm treatments were significantly greater than those in mice treated with vehicle (Figure 2A).

As the majority of women with endometriosis are of reproductive age and hormonally cycling (Dunselman et al. 2014;

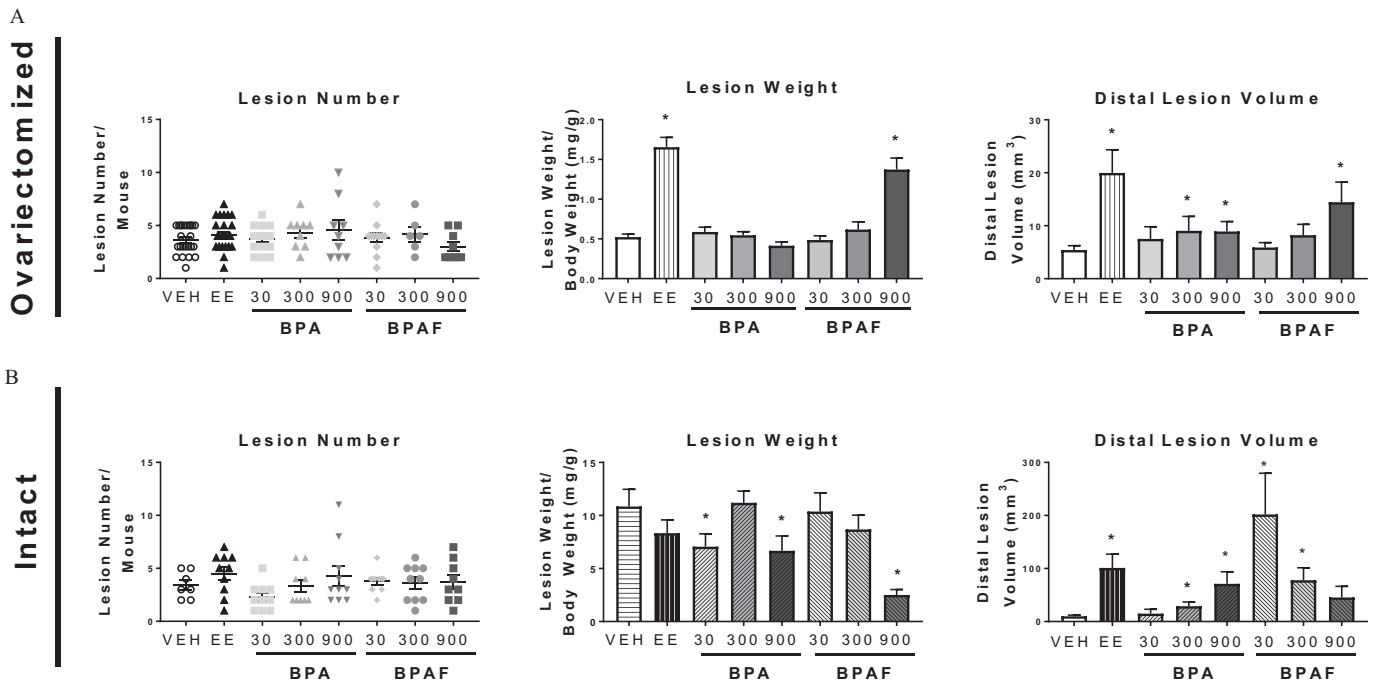

Figure 2. Lesion characteristics of bisphenol A (BPA) and bisphenol AF (BPAF) treated mice. (*A*) Lesion number, pooled lesion weight, and individual distal lesion volume from ovariectomized mice treated with vehicle (veh) ($n = 25$), Ethinylestradiol (EE) ($n = 25$), BPA 30 ($n = 15$), 300 ($n = 9$), and 900 ppm ($n = 9$), and BPAF 30 ($n = 12$), 300 ($n = 6$), and 900 ppm ($n = 9$). (*B*) Lesion number, pooled lesion weight, and individual distal lesion volume of intact mice treated with vehicle ($n = 7$), EE ($n = 10$), BPA 30 ($n = 10$), 300 ($n = 9$), 900 ppm, ($n = 10$) and BPAF 30 ($n = 10$), 300 ($n = 10$), 900 ppm ($n = 9$). A minimum of $n = 6$ mice (biological replicates) were used for each treatment from a minimum of three experimental replicates. $^{*}p < 0.05$, one-way ANOVA, followed by one-tailed *t*-test comparing treatment to vehicle. Error bars represent the standard error of the mean (SEM).

Messinis et al. 2014; Zullo et al. 2017), we next examined the effects of BPA or BPAF on endometriosis lesion characteristics in hormonally intact mice: lesion number, pooled lesion weight, and distal lesion volume. As in the ovariectomized model, the total lesion numbers in the intact model did not differ regardless of treatment or dose of EDC (Figure 2B). EE treatment did not affect total lesion weight; however, significantly lower lesion weight was observed with BPA 30 ppm and 900 ppm and BPAF 900 ppm treatments in comparison with vehicle treatment (Figure 2B). However, distal lesion volumes were significantly greater in mice treated with EE, BPA 300 ppm and 900 ppm, and BPAF 30 ppm and 300 ppm treated in comparison with those animals treated with vehicle (Figure 2B).

Lesion Gene Expression

RNA was isolated from all types of lesions located throughout the peritoneal cavity in each treatment group to examine the effect of BPA or BPAF on gene expression in endometriosis lesions. Known E_2/ERa-mediated targets, progesterone receptor (*Pgr)* and lactoferrin (*Ltf*) (Burns et al. 2012) were analyzed. In lesions from ovariectomized mice, as expected with EE treatment, expression of both *Pgr* and *Ltf* was significantly greater than in vehicle treatment (Figure 3A). BPA 300 ppm treatment resulted in significantly lower *Pgr* expression than vehicle treatment (Figure 3A). BPA 300 ppm and BPAF 300 and 900 ppm treatment groups exhibited significantly higher *Ltf* gene expression in comparison with vehicle treatment (Figure 3A). We further examined expression levels of genes known to be involved in endometriosis (Bukulmez et al. 2008; Bulun et al. 2012; Chang et al. 2011; Osteen et al. 2003): Estrogen Receptor 1 and 2 (*Esr1, Esr2*), Matrix Metallopeptidase 9 (*Mmp9*), Matrix Metallopeptidase 7 (*Mmp7*), and Mucin 4 (*Muc4*) (Figure 3A). Overall, *Esr1* and *Mmp9* expression did not differ with treatment. *Mmp7* expression was significantly lower with EE and BPAF 900 ppm, whereas BPA 300 ppm and BPAF 300 ppm treatments both exhibited significantly higher gene expression in comparison with vehicle treatment, in a nonmonotonic dose response. Expression of *Esr2* was below the level of detection in the majority of lesions from vehicle-treated mice, and significantly higher in all other treatment groups. When comparing *Esr2* expression to EE, only lesions from mice treated with BPAF 300 ppm had significantly higher expression of *Esr2*. A recently reported Tox21 Study on BPA analogs showed that these analogs, with BPAF in particular, activated the Constitutive Androstane Receptor (NR1I3) pathway (Pelch et al. 2017); therefore, we examined *NR1I3*, Flavin Containing Monooxygenase 5 (*Fmo5*), and C-C Motif Chemokine Ligand 2 (*Ccl2*) (Kiyosawa et al. 2008). *NR1I3* expression was not different with EE treatment but was significantly higher in the BPA 900 ppm and BPAF 30 ppm treatment groups in comparison with vehicle treatment (Figure 3A). Overall, *Fmo5* showed no significant differences (Figure S1A). The expression of *Ccl2* was significantly lower in EE and BPAF 900 ppm treatment groups but significantly higher in BPA 30 and 300 ppm, and BPAF 30 and 300 ppm in comparison with vehicle treatment.

To examine the effect of BPA or BPAF on gene expression in lesions from intact mice, RNA was isolated from all lesions located throughout the peritoneal cavity from each treatment group. The same genes as above were analyzed. *Pgr* and *Ltf* expression was significantly higher following EE treatment but did not differ after BPA or BPAF treatment in comparison with vehicle treatment (Figure 3B). Expression of *Esr1* was significantly higher in EE and BPAF 900 ppm treated mice in comparison with vehicle-treated mice. *Esr2* expression was significantly lower with BPAF 900 ppm treatment in comparison with vehicle-treated mice. Expression of *Mmp9* was significantly lower with EE, BPA 900 ppm, and BPAF 900 ppm in comparison with vehicle treatment. Last, *Mmp7, Muc4, NR1I3, Fmo5*, and *Ccl2* expression levels showed no

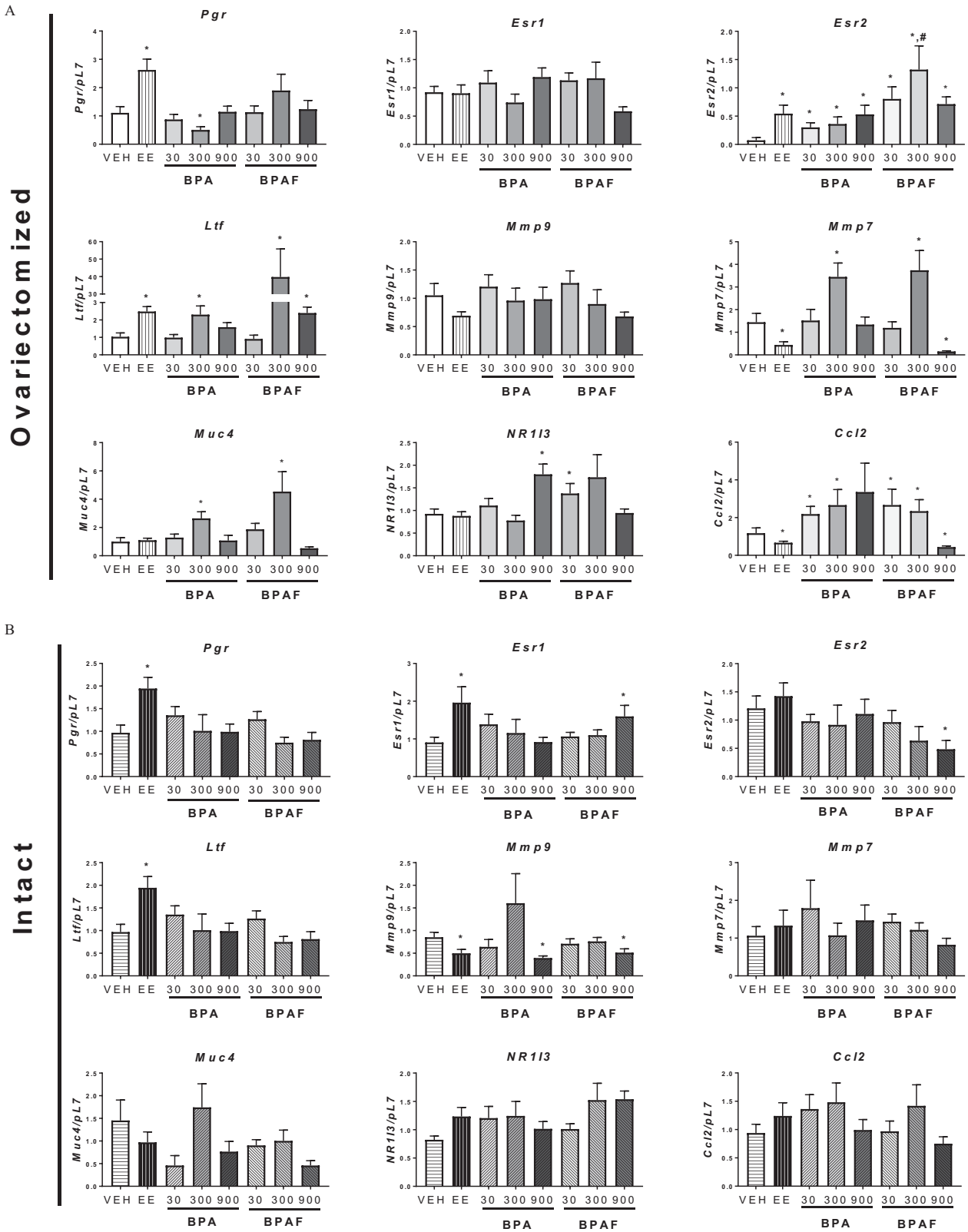

Figure 3. Lesion gene expression of bisphenol A (BPA) and bisphenol AF (BPAF) treated mice. (*A*) Lesion gene expression of *Pgr*, *Esr1*, *Esr2*, *Ltf*, *Mmp9*, *Mmp7*, *Muc4*, *NR1I3*, and *Ccl2* from ovariectomized mice treated with vehicle (veh) ($n = 11$ from 11 mice), Ethinylestradiol (EE) ($n = 14$ from 14 mice), BPA 30 ($n = 11$ from 9 mice), 300 ($n = 12$ from 9 mice), and 900 ppm ($n = 12$ from 9 mice), or BPAF 30 ($n = 11$ from 10 mice), 300 ($n = 11$ from 10 mice), and 900 ppm ($n = 13$ from 10 mice). (*B*) Lesion gene expression of *Pgr*, *Esr1*, *Esr2*, *Ltf*, *Mmp9*, *Mmp7*, *Muc4*, *NR1I3*, and *Ccl2* from intact mice treated with veh ($n = 11$ from 6 mice), EE ($n = 15$ from 8 mice), BPA 30 ($n = 9$ from 7 mice), 300 ($n = 6$ from 6 mice), 900 ppm ($n = 14$ from 9 mice) or BPAF 30 ($n = 15$ from 8 mice), 300 ($n = 12$ from 7 mice), 900 ppm ($n = 10$ from 5 mice) were used for each treatment. All gene expression data was normalized to pL7, and each gene was graphed relative to veh. *$p < 0.05$, one-way ANOVA, followed by one-tailed *t*-test comparing treatment to vehicle. #$p < 0.05$, one-way ANOVA, followed by one-tailed *t*-test comparing treatment to EE. Error bars represent the standard error of the mean (SEM).

statistical differences across the treatment and dose groups (Figure 3B, Figure S1B).

Lesion Proliferation and Apoptosis Profiles

Endometriosis lesions located throughout the peritoneal cavity were examined by histology to confirm the presence of endometriosis as defined by an endometriosis lesion being structurally organized, having distinct stromal and epithelial areas, and having hemosiderin-laden macrophages (Hsu et al. 2010). Estrogen stimulates uterine proliferation (Hewitt and Korach 2011); therefore, we examined the effect of BPA or BPAF treatment on lesion proliferation by Ki67 immunohistochemistry. Ovariectomized mice treated with EE exhibited significantly greater proliferation of the epithelium in the endometriosis lesions in comparison with vehicle treatment, as expected (Figure 4A, C). As with treatment with EE, treatment with BPAF 900 ppm resulted in significantly greater epithelial proliferation in endometriosis lesions of ovariectomized mice in comparison with vehicle treatment (Figure 4A, C). Lesion proliferation of mice treated with BPA 30, 300, and 900 ppm and BPAF 30 and 300 ppm were similar to vehicle treatment (Figure 4A, C). Immunoglobulin controls of serial sections remained unstained (Figure 4E).

In intact mice, the lesions exhibited no significant differences across treatment or dose groups for epithelial proliferation (Figure 4B, D). Because estrogenic EDCs are known to differentially affect proliferation or apoptosis at different concentrations (Moore et al. 2007), we stained lesions for cleaved caspase 3 to examine apoptosis. In ovariectomized mice, epithelial apoptosis did not significantly differ in the lesions in comparison with vehicle treatment (Figure S2A, C). Likewise, in intact mice, epithelial apoptosis did not significantly differ in the lesions in comparison with vehicle treatment (Figure S2B, D). Immunoglobulin controls of serial sections remained unstained (Figure S2E).

Estrous Cyclicity, Ovarian Gene Expression, and Ovarian Function

Although ovariectomy rids the mice of endogenous hormones and allows for the examination of a single hormone or EDC treatment without confounding endogenous hormones, women in general of child-bearing age and women with endometriosis of child-bearing age, are typically cycling (Dunselman et al. 2014; Messinis et al. 2014; Soliman et al. 2018; Zullo et al. 2017). Therefore, to more fully characterize the effects of BPA and BPAF on hormonal cyclicity and, consequently, indirect effects on endometriosis lesion growth, we examined estrous cyclicity. Mice in the vehicle group cycled normally through the four stages of the estrous cycle (Figure 5A). Treatment with EE significantly lengthened the estrous cycle, hence decreasing the number of cycles observed (Figure 5A). BPA 30, 300, and 900 ppm and BPAF 30 and 300 ppm did not significantly affect estrous cyclicity (Figure 5A). BPAF 900 ppm-treated mice were acyclic (Figure 5A). A graphical representation of the cycles of two representative mice in each treatment group over an 8-d period can be seen in Figure S3. Microscopic analysis of vaginal smears showed BPAF 900 ppm mice remained in metestrus/estrus throughout the 2 wk of vaginal smearing (Figure S4).

To determine the potential effects of BPA and BPAF on the ovaries of the intact mice, the ovary weight was normalized to mouse body weight. In comparison with ovarian weight in mice with vehicle treatment, ovarian weight was significantly higher in mice treated with BPA 300 ppm and significantly lower in the mice treated with BPAF 900 ppm (Figure 5B). After examining the ovarian weight, ovarian morphology was examined. Each ovarian section was examined to count the number of primordial follicles, primary follicles, secondary follicles, ovulatory and antral follicles, corpus luteum, and dead or atretic follicles (Figure S5). Across EE, BPA, and BPAF treatment groups in comparison with vehicle treatment, no significant differences in primordial follicle, primary follicle, or ovulatory and antral follicle counts were observed (Figure 5C). When examining the number of secondary follicles, significantly fewer were observed in the BPA 300 ppm and BPAF 900 ppm treatment groups in comparison with vehicle treatment (Figure 5E). For the corpus luteum, significantly more corpora lutea were observed in the BPA 30 ppm treatment group, and significantly fewer were observed in the BPAF 900 ppm group in comparison with vehicle treatment (Figure 5G). Last, when examining the number of dead or atretic follicles, a significantly greater number were observed in EE, BPA 30, 300, and 900 ppm, and in BPAF 30, 300, and 900 ppm treatment groups in comparison with vehicle treatment (Figure 5H).

We next examined the expression of genes responsible for steroidogenesis. Ovarian RNA was isolated to examine the effect of BPA or BPAF on gene expression. First, a known E2 target and important regulator in ovarian function, *Pgr*, was analyzed. As expected with the EE treatment, expression of *Pgr* was significantly higher relative to vehicle-treated mice. When comparing the BPA 30, 300, and 900 ppm, and BPAF 30, 300, and 900 ppm treatment groups to the vehicle-treated group, *Pgr* expression was significantly greater in all treatment groups (Figure 6A). Further gene expression analysis of key targets in the estrogen and ovarian steroidogenesis pathways (Bloom et al. 2016; Jones and Lopez 2014; Kiyosawa et al. 2008) were examined to assess the effects of BPA or BPAF on ovarian steroidogenesis. In these pathways, ovarian gene expression of Luteinizing Hormone/Choriogonadotropin Receptor (*Lhcgr*), Steroidogenic Acute Regulatory Protein (*Star*), Cytochrome P450 Family 11 Subfamily A Member 1 (*Cyp11a1*), Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- and Steroid Delta Isomerase 1 (*Hsd3b1*), Cytochrome P450 Family 17 Subfamily A Member 1 (*Cyp17a1*), Follicle Stimulating Hormone Receptor (*Fshr*), Cytochrome P450 Family 19 Subfamily A Member 1 (*Cyp19a1*), and Hydroxysteroid 17-Beta Dehydrogenase 1 (*Hsd17b1*) were examined (Figure 6A). Overall, the expression of *Lhcgr* and *Hsd3b1* were unaffected with treatments when compared with vehicle treatment (Figure 6A). *Star* and *Cyp11a1* gene expression were significantly lower in the BPA 30, 300, and 900 ppm, and BPAF 30, 300, and 900 ppm treatment groups in comparison with vehicle (Figure 6A). *Hsd17b1* expression was significantly lower with BPA 30 ppm treatment but significantly higher with BPA 300 ppm treatment in comparison with vehicle treatment. *Fshr* expression was significantly higher in comparison with vehicle treatment in the EE and BPA 300 and 900 ppm treatment groups (Figure 6A). Last, the gene expression of *Cyp19a1* and *Cyp17a1* were only significantly higher in the BPA 300 ppm treatment group in comparison with vehicle treatment (Figure 6A).

Hormone Levels in BPA- and BPAF-Treated Intact Mice

Estrous cyclicity is governed by hormonal regulation and feedback mechanisms through estrogen, luteinizing hormone, follicle stimulating hormone, and progesterone (Vadakkadath Meethal and Atwood 2005). We hypothesized that BPA and BPAF would alter normal circulating levels of these hormones, in particular progesterone and estradiol, because BPAF 900 completely abrogated estrous cyclicity and both BPA and BPAF significantly altered gene expression profiles in the steroidogenic enzyme pathways of progesterone, estrogen, and follicle-stimulating hormone. Overall, we found no significant differences in estradiol or follicle-stimulating hormone levels (Figure 6B). The levels of progesterone were significantly lower in the EE and BPAF

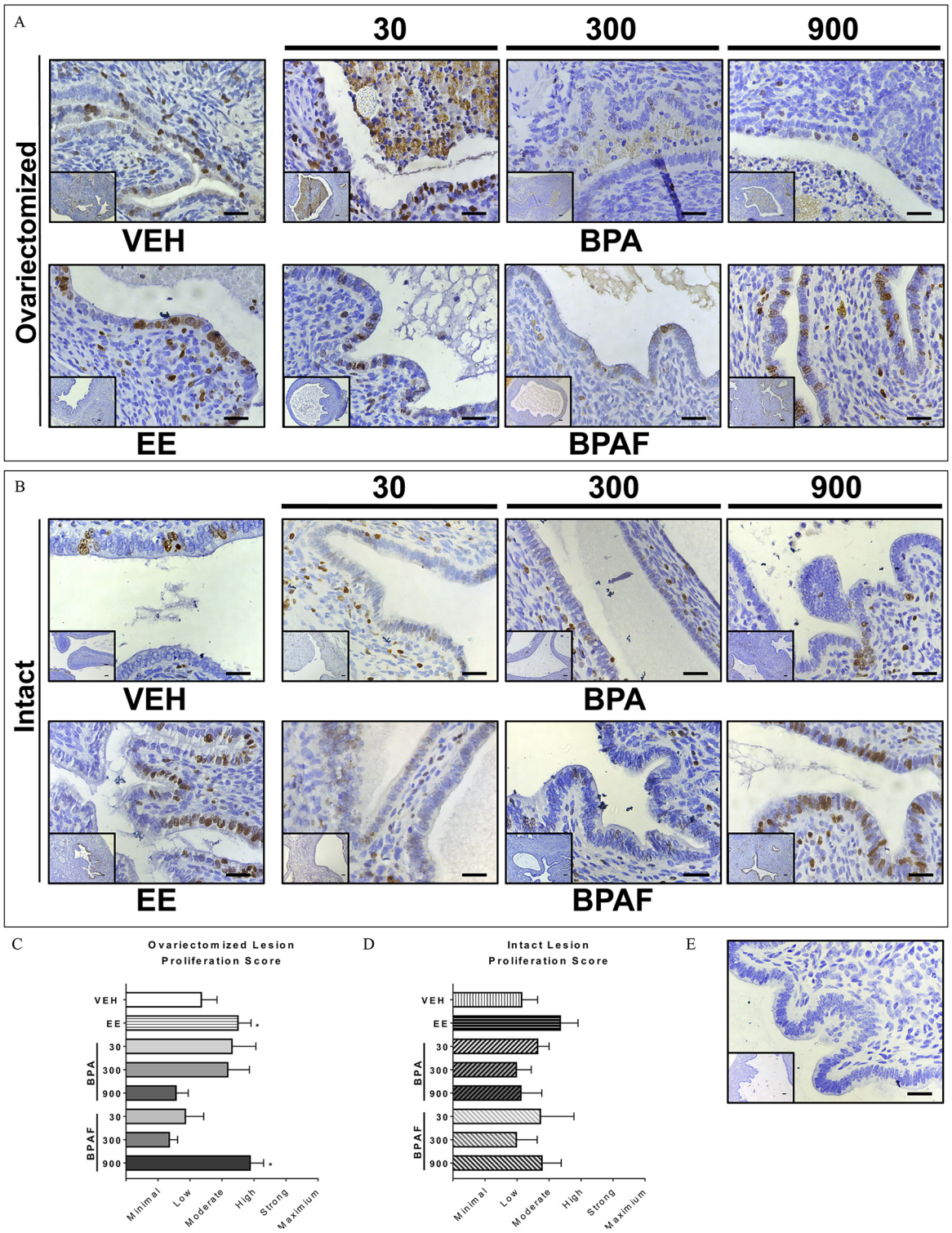

Figure 4. Lesion proliferation in bisphenol A (BPA) and bisphenol AF (BPAF) treated mice. (*A*) Representative photomicrographs of Ki67 staining in endometriosis lesions (400x, 100x inset) from ovariectomized mice treated with vehicle (veh) ($n = 8$), Ethinylestradiol (EE) ($n = 12$), BPA 30 ($n = 3$), 300 ($n = 5$), or 900 ppm ($n = 3$), or BPAF 30 ($n = 4$), 300 ($n = 4$), 900 ppm ($n = 5$) for 6 wk. (*B*) Representative photomicrographs of Ki67 stained endometriosis lesions (400x, 100x inset) from intact mice treated with veh ($n = 6$), EE ($n = 8$), BPA 30 ($n = 3$), 300 ($n = 6$), or 900 ppm ($n = 7$), or BPAF 30 ($n = 4$), 300 ($n = 5$), 900 ppm ($n = 5$). Ki67 stained sections were scored by individuals blinded to the treatment of the animal and reported for endometriosis lesions from (*C*) ovariectomized mice, and (*D*) intact mice, based on the observed percentage of positively stained epithelial cells with the following scale of minimal (0–10%), low (11–30%), moderate (31–50%), high (51–70%), strong (71–90%), or maximum (91–100%). For each category, a numerical value was assigned. Each sample was scored by RLJ, SAL, ADG, and KAB; these four scores were averaged to obtain a final score for each sample. The final scores of all samples were then averaged to obtain the final overall score for each treatment group. (*E*) Representative photomicrograph of negative control immunoglobulin stained endometriosis lesions (400x, 100x inset). Scale bars: 400x = 50 μm, 100x inset = 25 μm. $*p < 0.05$, one-way ANOVA, followed by one-tailed *t*-test comparing treatment to vehicle. Error bars represent the standard error of the mean (SEM).

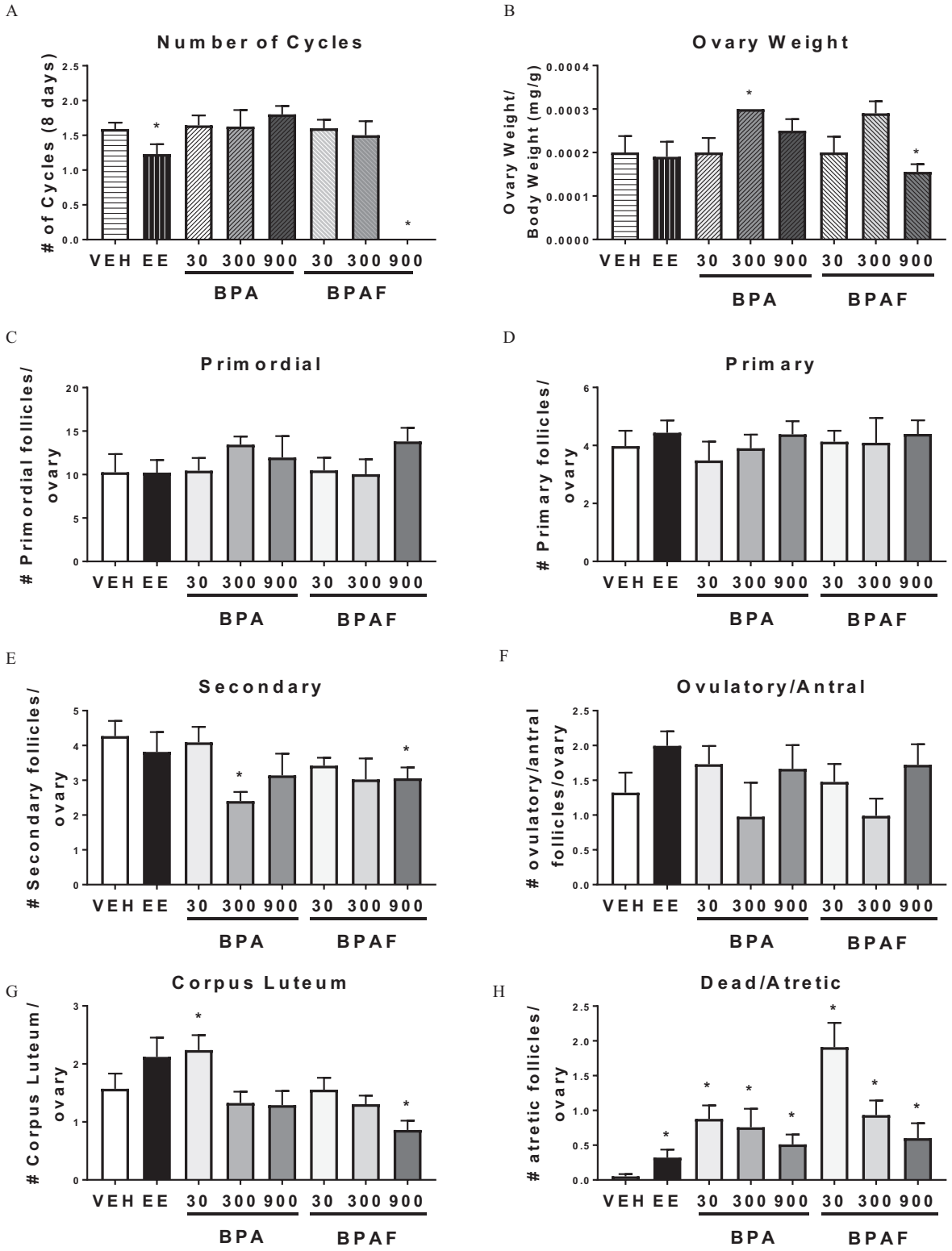

Figure 5. Estrous cyclicity patterns and ovarian growth. (*A*) Estrous cyclicity of intact mice treated with vehicle (veh) ($n = 7$), Ethinylestradiol (EE) ($n = 10$), bisphenol A (BPA) 30 ($n = 10$), 300 ($n = 10$), 900 ppm, ($n = 10$) and bisphenol AF (BPAF) 30 ($n = 10$), 300 ($n = 10$), 900 ppm ($n = 9$). (*B*) Ovary weight normalized to body weight of intact mice treated with vehicle ($n = 10$), EE ($n = 10$), BPA 30 ($n = 8$), 300 ($n = 8$), 900 ppm ($n = 8$), and BPAF 30 ($n = 11$), 300 ($n = 9$), 900 ppm ($n = 9$). (*C*) Number of primordial follicles; (*D*) primary follicles; (*E*). secondary follicles; (*F*) ovulatory/antral follicles; (*G*) corpus luteum; (*H*) dead/atretic follicles in 5 μm serial sections from vehicle ($n = 10$), EE ($n = 10$), BPA 30 ($n = 8$), 300 ($n = 8$), 900 ppm, ($n = 8$) and BPAF 30 ($n = 11$), 300 ($n = 9$), 900 ppm ($n = 9$) treated mice. *$p < 0.05$, one-way ANOVA, followed by one-tailed *t*-test comparing treatment to vehicle. Error bars represent the standard error of the mean (SEM).

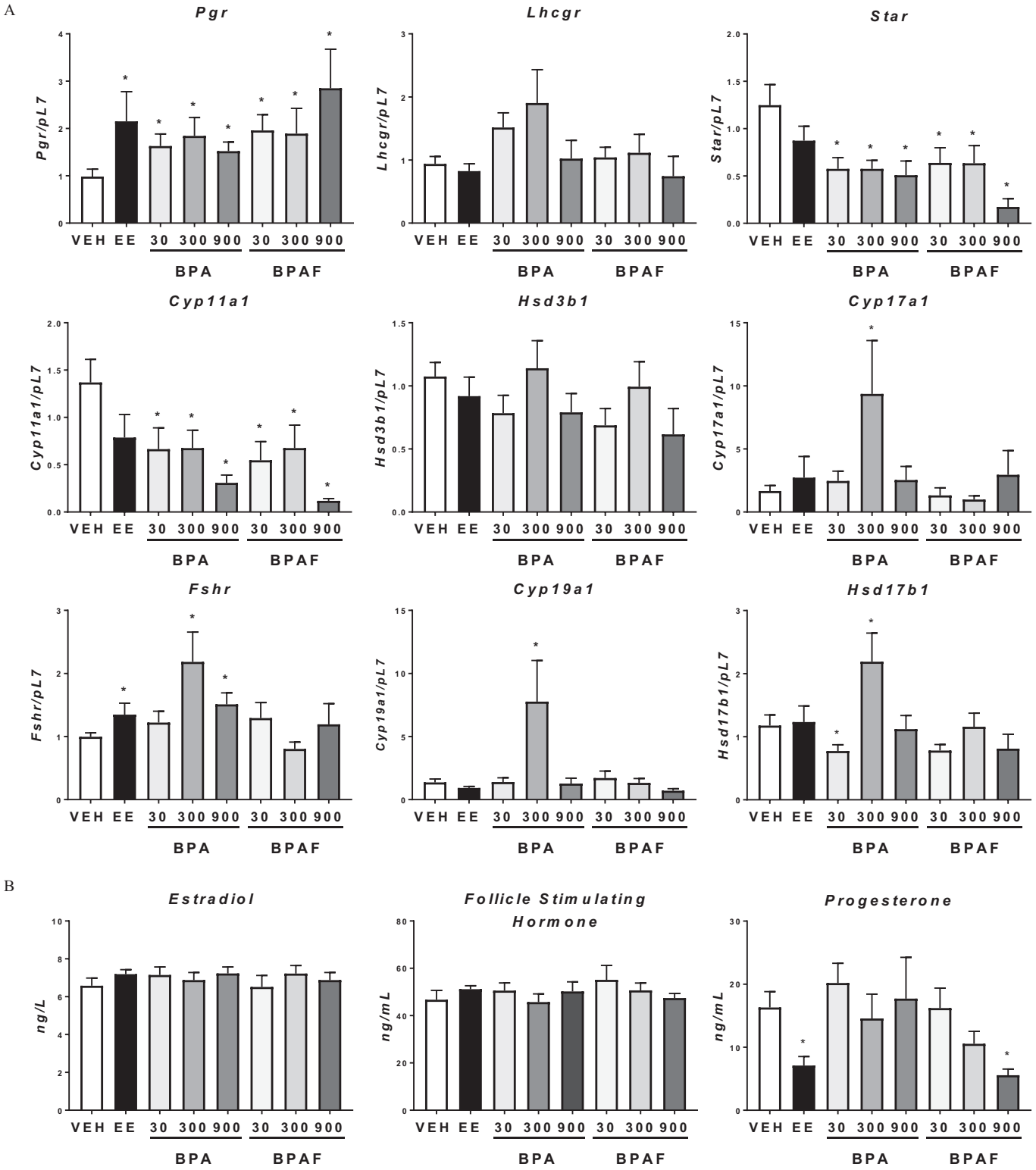

Figure 6. Ovarian gene expression and plasma hormonal analysis of bisphenol A (BPA) and bisphenol AF (BPAF) treated mice. (*A*) Ovarian gene expression of *Pgr*, *Lhcgr*, *Fshr*, *Cyp11a1*, *Star*, *Cyp19a1*, *Cyp17a1*, *Hsd3b1*, and *Hsd17b1* from mice treated with vehicle (veh) ($n = 14$), Ethinylestradiol (EE) ($n = 9$), BPA 30 ($n = 9$), 300 ($n = 9$), 900 ppm ($n = 9$), and BPAF 30 ($n = 8$), 300 ($n = 10$), 900 ppm ($n = 8$). B. Levels of estradiol (ng/L), follicle stimulating hormone (ng/mL), and progesterone (ng/mL) in plasma from mice treated with vehicle ($n = 14$), EE ($n = 8$), BPA 30 ($n = 9$), 300 ($n = 8$), 900 ppm ($n = 8$), and BPAF 30 ($n = 8$), 300 ($n = 9$), 900 ppm ($n = 8$). *$p < 0.05$, one-way ANOVA, followed by one-tailed *t*-test comparing treatment to vehicle. Error bars represent the standard error of the mean (SEM).

900 ppm treatment groups in comparison with vehicle treatment (Figure 6B).

Discussion

Epidemiological evidence demonstrates that BPA is found ubiquitously in the urine or serum of up to 93% of the population (Calafat et al. 2008; Vandenberg et al. 2007, 2010a, 2013). Although many people are exposed to BPA, the epidemiological evidence linking BPA to endometriosis is mixed (Anger and Foster 2008; Buck Louis et al. 2013). Cobellis et al. found that women with endometriosis had BPA in their urine and that the concentrations were significantly higher than in women without endometriosis (Cobellis et al. 2009). In more recent literature, nonovarian pelvic endometriosis was found to be associated with higher urinary concentrations of BPA, with an odds ratio (OR) of 3.0 (95% CI: 1.1, 7.6) (Upson et al. 2014). Additionally, similar findings from the Endometriosis: Natural History, Diagnosis, and Outcomes (ENDO) Study also suggested BPA was associated with higher incidence of endometriosis (Adjusted OR: 1.82, 95% CI: 1.01, 3.36) (Buck Louis et al. 2013). In mice, BPA elicited a uterotrophic response (Hewitt and Korach 2011; Markey et al. 2001), gestational BPA exposure altered adult murine uterine responses (Jorgensen et al. 2016) and has the potential to alter human uterine responses (Shelby 2008), and, as we previously showed through *in vitro* experiments, BPA and BPAF are EDCs that signal through the ERs with BPAF activating ER to a greater extent than BPA does (Li et al. 2012, 2013, 2018). Very limited information, let alone studies, are available for BPAF, but a study *in vivo* in zebrafish showed BPAF had higher toxicity and estrogenic activity than BPA did (Moreman et al. 2017). Because BPAF has been a replacement substituted into BPA-free products (D Chen et al. 2016), and, to date, no epidemiological studies have addressed exposure to BPAF and endometriosis association, BPAF was a critical part of our study. Here, we examined the effects of BPA or BPAF at increasing doses on the development and potentiation of endometriosis using both ovariectomized and hormonally intact mice.

Although humans and nonhuman primates are the only two species that menstruate, have an open reproductive system, and develop endometriosis spontaneously (Yamanaka et al. 2012), our model of endometriosis recapitulates human disease. We mimic human disease by injecting minced uterine tissue into the peritoneal cavity and allow this tissue to attach unassisted. Mouse lesions are found at sites similar to human lesion sites, are responsive to hormones, express a molecular signature comparable with that of human lesions, are organized (with glands, epithelium, and stroma), have smooth muscle organization, and have the infiltration of hemosiderin ladened macrophages (Burns et al. 2012, 2018). Recently, we suggested that two distinct phases of endometriosis exist: the initial phase of endometriosis that is immune predominant and the progression or maintenance phase of endometriosis that is hormone predominant (Burns et al. 2018). This idea was based on our previous work (Burns et al. 2018), which suggested that the attachment of menstrual tissue to form lesions is immune mediated, whereas the progression/maintenance phase, necessary for growth and secretions, is hormonally regulated. In the present study, we showed that BPA and BPAF were neither required for attachment nor acted as immunotoxicants because the same number of lesions were formed regardless of treatment in the presence or absence of hormones. Rather, BPA and BPAF played roles during the progression of endometriosis but did so differently based on the presence or absence of hormones.

To our knowledge, for the first time, we examined the estrogenic effects of BPA or BPAF on endometriosis by utilizing two different hormonal states for the first time. We examined the direct effects of these toxicants on endometriosis using ovariectomized animals with no endogenous hormones and on intact mice with endogenous circulating hormones. Divergent responses with BPA or BPAF acting as both agonists and antagonists on endometriosis lesions were observed based on the hormonal status of the mice. Doses of BPA and BPAF were chosen to represent the NOAEL (5 mg/kg/d) and LOAEL (50 mg/kg/d) set by the EPA for BPA (as no NOAEL or LOAEL are set for BPAF) (EPA 2010), the range of human exposure 0.1–86 mg/kg/d (Vandenberg et al. 2007, 2010a, 2010b, 2013, vom Saal et al. 2007, 2010), and the maximum consumption of BPA denoted by the World Health Organization (WHO) of 120 mg/kg/d (WHO 2009). In ovariectomized mice, treatment with BPA at lower (300 ppm) and higher (900 ppm) than LOAEL and treatment with BPAF 900 ppm resulted in larger distal lesion volume in comparison with treatment with vehicle. On the other hand, in hormonally intact mice with endometriosis, treatment with BPA at lower (300 ppm) and higher (900 ppm) than LOAEL resulted in greater distal lesion volume, and treatment with BPAF at 30 ppm and 300 ppm resulted in significantly greater distal lesion volume in comparison with treatment with vehicle.

When examining the role of proliferation and apoptosis in lesions and uteri, classically, estrogen increases uterine proliferation and water imbibition to increase epithelial cell height and uterine weight, respectively (Hewitt and Korach 2011). Hewitt and Korach demonstrated that BPA, in many respects, mimicked a weak estrogen (estriol) in response to weight and gene expression differences 2 h post treatment, but BPA did not fully mimic a strong estrogen (estradiol) 24 h post treatment in ovariectomized mice (Hewitt and Korach 2011). In our lesions, higher epithelial proliferation scores were observed from chronic EE and BPAF 900 ppm treatment. Lesions from ovariectomized mice demonstrated, as expected, low apoptotic scores. In intact mice, however, the complexity of the interaction of BPA or BPAF with endogenous hormones became more apparent with these toxicants as no differences in proliferation or apoptotic scores were observed.

The complexity of toxicant effect in the presence or absence of hormones continued when examining gene expression in the endometriosis lesions. In lesions from ovariectomized mice, as expected from estrogenic EDCs, high doses of BPA or BPAF showed significantly greater expression of known ER target genes; however, in lesions from hormonally intact mice, BPA or BPAF did not elicit the same estrogenic-mediated response. Although the etiology of endometriosis is not fully defined, estrogen is a known driver of disease; therefore, we examined genes from pathways that are hypothesized to play a role in endometriosis. Unfortunately, in the presence of endogenous hormones, those genes were similarly not altered by BPA or BPAF in the lesions, which may be because the lesions were established and not likely actively remodeling at this stage. Based on a recent study by Pelch et al., in hormone-free cell culture conditions, BPA and BPAF were predicted to signal through the nuclear receptor *NR1I3* (Pelch et al. 2017); therefore, we examined *Nr1I3* target genes. We observed *NR1I3*-mediated signaling in lesions from ovariectomized mice treated with BPA or BPAF, but this response was not seen in lesions from intact mice, suggesting other pathways are involved and that circulating hormones are master regulators. *In vitro* BPA acted more agonistically on *NR1I3*, whereas BPAF acted more antagontiscally (Pelch et al. 2017). In the absence of hormones, we observed similar findings as *NR1I3* gene expression was significantly higher at the highest dose of BPA 900 ppm as well as BPAF 30 ppm in comparison with those treated with vehicle. In the presence of endogenous

hormones, *NR1I3* was not different in any of the treatment groups in comparison with vehicle treatment. These results overall suggested that through the proposed mechanisms of estrogen-mediated signaling, BPA and BPAF on some target genes, in the absence of hormones, especially BPAF 900 ppm, are acting estrogenically in comparison with vehicle treatment. However, in the presence of endogenous hormones, minimal ER-target genes were activated by BPAF 900 ppm acting estrogenically in comparison with vehicle treatment, which suggested competing roles for endogenous and exogenous xenoestrogens.

Oral contraceptive therapy is designed to abrogate menstrual cyclicity with low-dose therapy of 20 μg EE to high-dose therapy of 50 μg EE (Calhoun and Batur 2017). Although our dose of EE was low in comparison (0.01 ppm; approximately 800–2000 fold less than oral contraceptive therapy), we would predict a slight disruption in estrous cyclicity. Mice treated with EE were delayed in estrus, metestrus, and diestrus, typically an additional day; however, the dose of EE was not high enough to abrogate cyclicity in these mice. BPA 30, 300, and 900 ppm and BPAF 30 and 300 ppm did not alter estrous cyclicity; however, BPAF 900 ppm completely abrogated cyclicity as the mice persisted in a state of metestrus or estrus. As an abrogation or delay in cyclicity is quite detrimental to reproduction and often linked to disruptions in the hypothalamic gonadal axis (Toufexis et al. 2014), we examined ovarian and hormonal status after chronic BPA and BPAF exposure to determine if they correlated to lesion responses.

To our knowledge, this is the first study to dose adult cycling female mice to chronic BPA and BPAF treatment, and we carefully examined differences in ovarian morphology. We found no differences in primordial, primary, and ovulatory and antral follicles with treatment. BPA (2.5, 25, 250, 2,500, and 25,000 μg/kg bw/d) treatment dosed prenatally to 12 months of age in NCTR Sprague-Dawley rats, by oral gavage, showed results similar to ours with no differences in primordial, primary, or antral follicles at postnatal day 90 of continuous treatment (Patel et al. 2017). In our experiments, all treatment groups (EE, BPA 30, 300, and 900 ppm, and BPAF 30, 300, and 900 ppm), had significantly higher numbers of dead or atretic follicles. With the BPA and BPAF treatment groups, the lowest doses of the toxicants had greater effects on oocyte death, with a smaller effect as the dose of EDC became higher. These data suggested that BPA and BPAF, especially at environmentally relevant doses, were likely disrupting oocyte quality. Additionally, similar to reported murine prenatal exposure to BPA (Patel et al. 2017), the chronic BPA and BPAF exposure to adult mice in our study also disrupted normal ovarian function. This finding suggested that BPA and BPAF exposure adversely affected the ovary at doses lower and higher than the LOAEL, which could indirectly have a negative impact on endometriosis.

To determine if ovarian steroidogenesis and endogenous hormones were the cause of the differences in lesion responses after BPA and BPAF treatment, we examined gene expression of ovarian steroidogenic enzymes and hormone production. Alterations in gene expression of genes involved in the estrogen, progesterone, and follicle-stimulating hormone pathways in both monotonic and nonmonotonic dose responses led us to examine these circulating hormones in the mice. Both estrogen and follicle-stimulating hormone did not show any differences across treatments. Progesterone, however, was significantly lower in both EE and BPAF 900 ppm. With significantly lower levels of progesterone in the EE and BPAF 900 ppm treatment groups, we concluded that these treatments interfered with the hypothalamic gonadal axis by mimicking estrogen to chemically maintain lower levels of progesterone, which in turn disrupted uterine cyclicity. With maintained low levels of progesterone and higher levels of an estrogenic mimetic, we hypothesized that the dramatic threshold and subsequent withdrawal in progesterone needed for normal hypothalamic gonadal signaling does not occur; therefore, the mice remained in metestrus and ceased to cycle. Our results with high doses of BPAF were similar to results found with zebrafish (Shi et al. 2015) that showed high doses of BPAF disrupted the hypothalamic gonadal axis. Therefore, our findings further support the *in vivo* zebrafish study that BPAF had more toxicity than BPA.

Conclusions

BPA and BPAF acted differently on endometriosis lesions due to hormonal status. In ovariectomized mice, these EDCs stimulated lesion growth. In intact mice, when endogenous hormones were present, the behavior of these two EDCs was quite different. In our *in vivo* model of endometriosis, BPA and BPAF had the ability to potentiate endometriosis in intact mice as the distal lesion volumes were significantly higher. Although BPAF is being used as a substitute in place of BPA (D Chen et al. 2016), BPAF appeared to be as estrogenic as BPA, if not more estrogenic than BPA, and could have an impact on the environmental contribution to the higher incidence of endometriosis observed over the last 40 years.

Additionally, our study allowed us to examine ovarian and hormonal responses to chronic BPA and BPAF exposure in adult mice. These EDCs in our study altered various ovarian steroidogenic responses throughout the steroidogenesis pathways that are known to function in theca and granulosa cells (Bloom et al. 2016; Jones and Lopez 2014; Kiyosawa et al. 2008). Although only high doses of BPAF abrogated cyclicity, the higher incidence of atretic oocytes is particularly alarming, as all doses of toxicant affected oocyte quality. Together these data suggested that these toxicants may disrupt proper positive and negative feedback within the hypothalamic gonadal axis, which ultimately would affect the growth of endometriosis.

In conclusion, these findings furthered our understanding of the effects and hormonal impacts of BPA and BPAF on endometriosis perturbation in ovariectomized and hormonally intact mice. BPAF appeared to be as estrogenic as BPA, if not more estrogenic than BPA, and may contribute as an environmental toxicant to endometriosis incidence.

Acknowledgments

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